

# Early-phase Innate Immune Suppression in Murine Severe Sepsis Is Restored with Systemic Interferon- $\beta$

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## ABSTRACT

**Background:** Sepsis is a leading cause of death in the intensive care unit. Immune modulatory therapy targeting sepsis-associated proinflammatory responses has not shown survival benefit. Here, the authors evaluated innate immunity at the early stage of murine mild or severe peritoneal sepsis induced by cecal ligation and puncture, and the effect of systemic interferon- $\beta$ , a potent inflammatory mediator, on severe sepsis as well as its mechanism of action.

**Methods:** Mild and severe sepsis was induced in C57BL/6 mice by cecal ligation and puncture with 22- and 18-gauge needles for puncture, respectively. Interferon- $\beta$  (700 U/g) was subcutaneously administered either before or 12 h after cecal ligation and puncture for the severe sepsis group.

**Results:** Severe sepsis resulted in significantly lower 6-day survival rates than mild sepsis ( $n = 48$ , 25% *vs.*  $n = 11$ , 81.8%,  $P = 0.002$ ), significantly less phagocytic capacity of peritoneal exudate cells, and lower CXC chemokine receptor-2 expression on circulating neutrophils at 24 h after cecal ligation and puncture. Interferon- $\beta$  administration 12 h after cecal ligation and puncture associated with significantly improved survival ( $n = 34$ , 52.9%,  $P = 0.017$ ) increased the number and function of peritoneal exudate cells, peritoneal/systemic inflammatory cytokine/chemokine concentrations, and CXC chemokine receptor-2 on neutrophils, compared with the severe sepsis controls. However, those responses were not observed in the prophylactic interferon- $\beta$  group ( $n = 24$ ). Interferon- $\beta$  increased lipopolysaccharide-induced interleukin-6 messenger RNA/protein expression of lipopolysaccharide-tolerant murine peritoneal macrophages, which was not observed in nontolerant cells.

**Conclusions:** In severe sepsis, immune suppression occurs within 24 h and is associated with worse mortality. Interferon- $\beta$  given after the onset of peritonitis restores impaired innate immunity *in vivo* and *in vitro*. (**ANESTHESIOLOGY 2018; 129:131-42**)

ANNUALLY, 750,000 cases of sepsis are reported in the United States,<sup>1,2</sup> and this disease is associated with a sustained high mortality rate, ranging from 15 to 50%.<sup>3-9</sup> Death relates to multiple organ dysfunction that involves kidneys, liver, pancreas, adrenals, coagulation system, central nervous system, cardiovascular system, and lungs.<sup>10</sup> Acute respiratory distress syndrome is a major remote organ pathology accompanied by sepsis.<sup>11</sup>

Despite an increase in our knowledge of the pathogenesis of sepsis, all sepsis trials with immune-modulating drugs, most of which have targeted the sepsis-associated proinflammatory response, have reported no survival benefit.<sup>12-14</sup> One of the challenging issues for sepsis research is a heterogeneity of study populations; a vague definition of the disease accepts heterogeneity in microorganisms and the site of infection,<sup>13</sup> and genetic heterogeneity of hosts, which causes different responses to invading microorganisms.<sup>15</sup> Furthermore, immune status varies widely

### What We Already Know about This Topic

- Sepsis is a leading cause of death, and targeting sepsis-associated inflammatory responses has not been shown to prove beneficial.

### What This Article Tells Us That Is New

- In an established mouse model of sepsis (cecal ligation and perforation), severe *versus* mild sepsis was associated with increased mortality, less capacity of peritoneal inflammatory cells for phagocytosis, and decreased expression of focal and systemic cytokines and chemokine receptor expression on circulating neutrophils. These effects were reversed by the administration of the immune stimulant interferon- $\beta$  after—but not before—severe sepsis was established. Interferon- $\beta$  after the onset of peritonitis may restore impaired innate immunity and improve outcome.

in the critically ill, usually preceding a hyperimmune state followed by subsequent immune paralysis, particularly in patients

Corresponding article on page 5. Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site ([www.anesthesiology.org](http://www.anesthesiology.org)). Part of the work presented in this article has been presented at the American Thoracic Society International Conference 2013, Philadelphia, Pennsylvania, May 19, 2013, and at the American Thoracic Society International Conference 2014, May 18, 2014, San Diego, California.

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with severe sepsis.<sup>16,17</sup> This contributes to the heterogeneity of the study population. Therefore, individual assessment of the immune status of patients is required to identify the appropriate population for a particular immunomodulatory treatment.

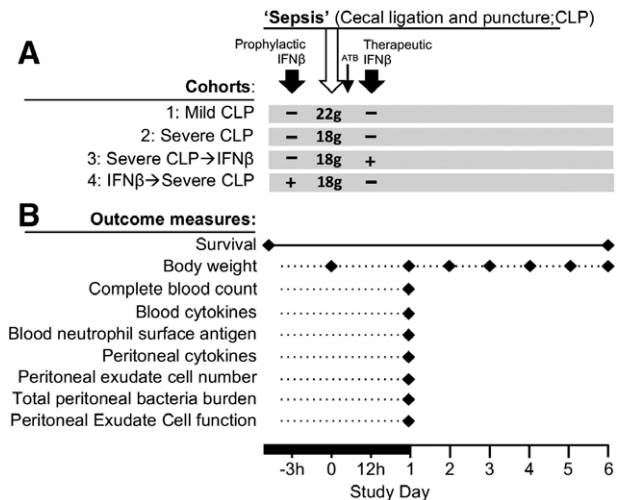
Previous studies have reported suppressed functions in innate immune cells of patients with sepsis that correlates with poor outcomes.<sup>17–22</sup> Phagocytic activity of neutrophils in patients with sepsis within 24 h after admission was impaired in patients with worse outcomes.<sup>19</sup> Previous publications indicate that impaired innate immunity is present in the early stages after infection in some populations of severe sepsis with worse outcomes, although no reports clearly show a relationship between sepsis severity and innate immune function, especially in the early stage of infection. Under such circumstances, it is worth noting that an open-label clinical trial of systemic interferon- $\beta$ , a type 1 interferon family member, significantly improved survival of patients with acute respiratory distress syndrome compared with a conventional treatment cohort. Seventy percent of recruited patients had infectious complications, suggesting that modulation of host-defense mechanisms might affect improved survival.<sup>23</sup> However, this has not been previously reported. We previously demonstrated impaired alveolar macrophage effector and regulatory functions in murine two-hit models (cecal ligation and puncture-induced peritoneal sepsis—*Pseudomonas aeruginosa* pneumonia), which is restored by systemic interferon- $\beta$ .<sup>24</sup>

Because interferon- $\beta$  is a potent inflammatory mediator and is highly induced by Toll-like receptor activators such as lipopolysaccharide,<sup>25</sup> it is anticipated that interferon- $\beta$  administration may be of therapeutic benefit to patients with sepsis, especially when their immune status is suppressed. Accordingly, this study evaluated innate immunity in the early stages of murine mild or severe peritoneal sepsis produced by cecal ligation and puncture and the survival rate from sepsis (primary outcome).<sup>26</sup> Furthermore, the effects of the systemic administration of interferon- $\beta$  on survival of severe sepsis as well as on immune functions were assessed.

## Materials and Methods

### Peritoneal Sepsis Models

All studies were conducted using procedures approved by the animal experimentation committee of the graduate school of medicine, the University of Tokyo, Tokyo, Japan (Med-P16-027). Male C57BL/6 mice, 8 to 10 weeks old (Jackson Laboratories, Japan), were used for all studies (fig. 1A). Cecal ligation and puncture using either a 22- or 18-gauge needle was used to induce mild peritoneal sepsis with anticipated low mortality rate (mild cecal ligation and puncture control group) or severe peritoneal sepsis with anticipated high mortality rate (severe cecal ligation and puncture control).<sup>27,28</sup> One milliliter of normal saline and imipenem/cilastatin (25 mg/kg) was subcutaneously administered shortly after the cecal ligation and puncture. An additional two groups of mice received interferon- $\beta$  (700 U/g; PBL Assay Science, USA)



**Fig. 1.** Establishment of low and high mortality peritoneal sepsis models and the effect of systemic interferon (IFN)  $\beta$  on severe sepsis. (A) Schematic illustration of mild or severe sepsis in mice induced by cecal ligation and puncture (CLP) and the cohorts studied. Mild (cohort 1) and severe (cohort 2 to 4) sepsis was caused by CLP induced by 22- and 18-gauge needles, respectively. All mice received 1 ml of phosphate buffered saline for hydration and broad-spectrum antibiotics (ATB) by subcutaneous injection within 1 h after CLP surgery. Phosphate buffered saline (cohorts 1, 2) and IFN $\beta$  (cohorts 3, 4) were administered subcutaneously in a volume of 200  $\mu$ l. In cohort 3, IFN $\beta$  was therapeutically administered 12 h after CLP. In cohort 4, IFN $\beta$  was prophylactically administered 3 h before CLP. (B) Outcome measurements were evaluated. Diamonds indicate the times specimens were obtained for the indicated studies.

subcutaneously either 3 h before (prophylactic interferon- $\beta$  administration: severe cecal ligation and puncture prophylactic interferon- $\beta$ ) or 12 h after the cecal ligation and puncture (therapeutic interferon- $\beta$  administration: severe cecal ligation and puncture therapeutic interferon- $\beta$ ; fig. 1A). Animals were kept in a cage with water and chow available *ad libitum*. The 6-day survival rate was evaluated for the four groups of mice that developed peritonitis. A pilot study indicated mice that survive the observation period (6 days) gained weight and recovered from peritonitis (Supplemental Digital Content 1, <http://links.lww.com/ALN/B676>).

### Peritoneal Innate Immunity

Another set of mice was euthanized by exsanguination of the neck vessels during isoflurane anesthesia at 24 h after cecal ligation and puncture, and peritoneal exudate cells were harvested with 5 ml  $\times$  2 of Roswell Park Memorial Institute medium supplemented with 1 mM of EDTA. Supernatants of lavage fluid were stored at  $-80^{\circ}\text{C}$  for cytokine quantification (fig. 1B). Then,  $2.5 \times 10^4$  peritoneal exudate cells in 100  $\mu$ l Roswell Park Memorial Institute medium were seeded onto 96-well cell culture plates and incubated for 2 h in a humidified incubator at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$  to allow cells to attach to the plate. Phagocytic capacity was evaluated using 50  $\mu\text{g/ml}$  pHrodo Green *Escherichia coli* BioParticles Conjugate (IncuCyte pHrodo

Green E. coli Bioparticles, Essen Bioscience, Inc., USA), and time-sequence phagocytosis was evaluated using the InCuCyte ZOOM Live-Cell Analysis System (Essen BioScience, Inc.) according to the manufacturer's instructions.

### Systemic Innate Immunity

Heparinized whole blood was obtained from mice in each group *via* the orbital vein 24 h after cecal ligation and puncture. Neutrophils in whole blood were identified by staining with fluorescein isothiocyanate-labeled Ly-6G antibody (Cat No. 130-093-138, Miltenyi Biotec K. K. Koto-ku, Japan), and chemokine receptor expression was evaluated with phycoerythrin-labeled CD182 (CXC chemokine receptor-2) antibody (Clone; SA044G4, Cat. No. 149304, BioLegend, USA) using flow cytometry (BD ACCURI C6, BD Biosciences, USA).

### In Vitro Host-defense Function of Peritoneal Macrophages

Phagocytic capacity of peritoneal macrophages harvested from normal mice were evaluated *in vitro*. First,  $5 \times 10^5$  cells of peritoneal macrophages/500  $\mu$ l of Roswell Park Memorial Institute medium were stimulated with lipopolysaccharide for various durations with or without interferon- $\beta$  and their phagocytic capacity was evaluated with fluorescent labeled opsonized latex beads (Cayman Chemical Co., USA) according to the manufacturer's instructions.

### Peritoneal/Serum Cytokines

Cytokines (interleukin-6, keratinocyte chemoattractant, monocyte chemoattractant protein-1, tumor necrosis factor- $\alpha$ , and interleukin-10) in peritoneal lavage supernatant, serum collected at 24 h after cecal ligation and puncture, and peritoneal macrophage media supernatant were evaluated with the BD Cytometric Bead Array Flex Set System (BD ACCURI C6, BD Biosciences) according to the manufacturer's instructions.

### mRNA Expression Assay

A murine peritoneal macrophage-derived cell line, RAW264.7 (American Type Culture Collection, USA) was stimulated with lipopolysaccharide with or without coinubation with 15,000 U/ml of interferon- $\beta$ , and the expressions of suppressor of cytokine signaling-3, p21, and IL-6 were quantified by TaqMan Gene expression assay (Applied Biosystems, USA) using the StepOne Real-Time PCR System (ThermoFisher Scientific, USA) according to the manufacturer's instructions. Expression values for each gene were calculated by normalization to glyceraldehyde-3-phosphate dehydrogenase, and fold change in expression to unstimulated controls was determined for each condition. Each assay identification number for TaqMan Gene expression assay was as follows: *Gapdh*; Mm99999915\_g1, *Il6*; Mm00446190\_m1, *Socs3*; Mm00545913\_s1, cyclin-dependent kinase inhibitor 1A (*p21*); Mm04205640\_g1. IL-6 secreted into the culture media was evaluated by enzyme-linked immunosorbent assay.

### Sample Size Estimation, Method to Randomize, and Data Handling

For survival experiments, we set the sample size to 20 based on past publications.<sup>28–30</sup> Sample size calculations revealed  $n = 23$  if we set an estimated survival for severe cecal ligation and puncture control and severe cecal ligation and puncture therapeutic interferon- $\beta$  as 15% and 55%, respectively, and  $\alpha$ -error = 0.05, power = 0.8. In response to reviewer suggestions, an additional experiment for severe cecal ligation and puncture control, severe cecal ligation and puncture therapeutic interferon- $\beta$ , and severe cecal ligation and puncture prophylactic interferon- $\beta$  was conducted ( $n = 10$  each). We did not apply strict randomization techniques to allocate mice into experimental groups. All cecal ligation and puncture procedures were performed by one surgeon blinded to the group allocation, and additional interventions to mice were determined at the beginning of experiments.

For time-sequence phagocytosis assays, two samples were excluded (one sample had initial high fluorescent intensity because of buffer conditions that prevented proper evaluation of phagocytosis, and another sample was from a mouse without designated surgery). Two peritoneal lavage samples were not obtained because of technical errors for cytokine evaluation.

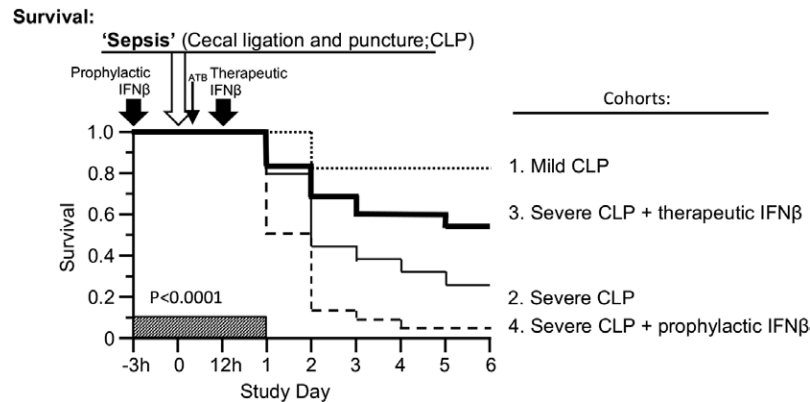
### Statistical Analyses

Normality and equal variance of all numerical data were assessed using the Kolmogorov-Smirnov test and Levene's median test, respectively. If normality and equal variance tests failed, a nonparametric rank-sum test was used for comparison. Data are the mean  $\pm$  SD for parametric values and the median (interquartile range) for nonparametric values. Survival rate (primary variable) was compared between treatment groups by the log rank test. Multiple group comparisons were performed by *post hoc* Tukey-Kramer honestly significant difference for parametric values and Dunn's signed rank-sum test for nonparametric values after one-way ANOVA. Statistical analyses were performed using JMP Pro 13.2.0 (SAS Institute Inc., USA) and SigmaPlot ver. 12.0 (Systat Software, Inc., USA).  $P$  values < 0.05 indicated statistical significance. Additional methodologic details are included in Supplemental Digital Content 2 (<http://links.lww.com/ALN/B677>).

## Results

### Therapeutic Efficacy of Interferon- $\beta$ on the Survival of Severe Peritoneal Sepsis Mice

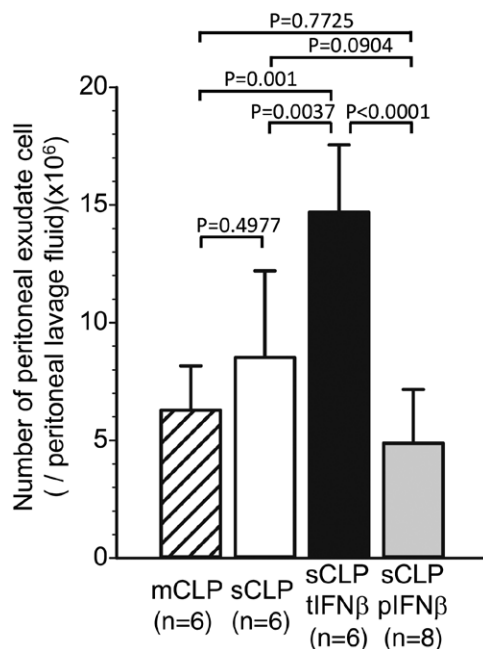
In our model of severe peritonitis induced by cecal ligation and puncture with an 18-gauge needle (severe cecal ligation and puncture control), the 6-day survival rate was 25%, whereas mild peritonitis induced with a 22-gauge needle (mild cecal ligation and puncture control) showed 82% survival. Systemically administered interferon- $\beta$  at 700 U/g 12 h after severe cecal ligation and puncture (severe cecal ligation and puncture therapeutic interferon- $\beta$ ) was associated with improved survival up to 53% (fig. 2). The survival rate was less than 5% in the prophylactic interferon- $\beta$  (severe cecal ligation and puncture prophylactic interferon- $\beta$ ) group (fig. 2).



**Fig. 2.** Mouse survival was affected by the severity of cecal ligation and puncture (CLP), and the timing of systemic interferon ( $\text{IFN}$ )  $\beta$  administration. Kaplan-Meier analysis of mice subjected to mild sepsis (cohort 1,  $n = 11$ ), severe sepsis control (cohort 2,  $n = 48$ ), severe sepsis and therapeutic  $\text{IFN}\beta$  (cohort 3,  $n = 34$ ), and severe sepsis with prophylactic  $\text{IFN}\beta$  (cohort 4,  $n = 24$ ). On day 6, survival in cohort 2 (25%) was significantly lower than in cohort 1 (81.8%; log-rank test,  $P = 0.002$ ). Compared with cohort 2, the survival of therapeutic  $\text{IFN}\beta$ -treated mice (cohort 3) was significantly improved (52.9%; log-rank test,  $P = 0.017$ ). In contrast, prophylactic  $\text{IFN}\beta$ -treated mice (cohort 4) had significantly lower survival rates than severe sepsis controls (4.1%; log-rank test,  $P = 0.001$ ). Data represent combined results from six separate survival experiments, each with similar results. ATB = antibiotics.

### Effects of Severe Sepsis on Primary Phagocyte Function in the Peritoneum and Therapeutic Efficacy of Interferon- $\beta$

The number of phagocytes recruited into the peritoneal cavity was significantly higher in the severe cecal ligation and puncture therapeutic interferon- $\beta$  group than the other three groups (fig. 3). However, the number of residual bacteria in the



**Fig. 3.** Numbers of peritoneal exudate cells are not reduced in severe (s) cecal ligation and puncture (CLP) control compared with mild (m) CLP and are significantly increased by therapeutic (t) interferon ( $\text{IFN}$ )  $\beta$ . Peritoneal lavage was performed 24 h after CLP with  $2 \times 5 \text{ ml}$  of Roswell Park Memorial Institute media supplemented with 1 mM Ethylene-Diamine-Tetra-Acetic acid. Numbers of harvested cells were counted using a hemocytometer. More than 99% of cells were viable by trypan blue staining. Numbers (n) of mice included for each group are indicated in parentheses.

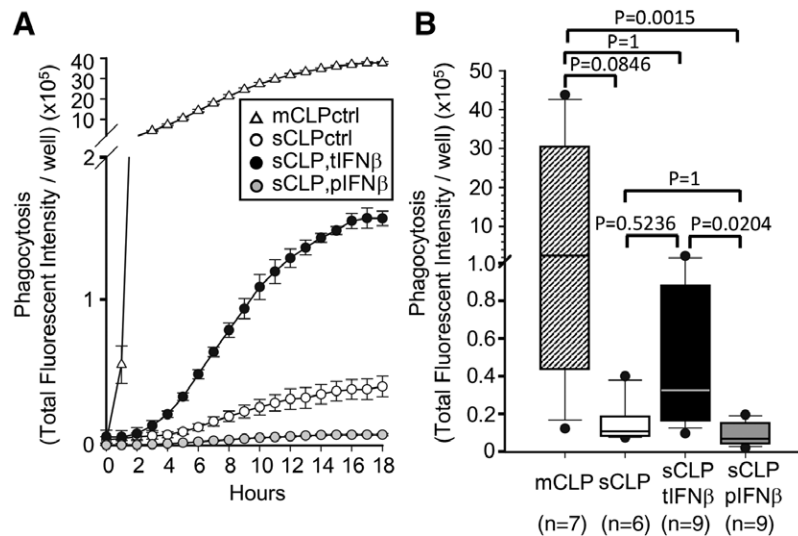
peritoneal cavity was not significantly different among groups with severe cecal ligation and puncture (Supplemental Digital Content 3, <http://links.lww.com/ALN/B678>). Although numbers of recovered cells by peritoneal lavage were comparable between severe and mild cecal ligation and puncture controls (fig. 3, greater than 99% of cells were alive by trypan blue staining, greater than 60% were neutrophils assessed by differential counts with Diff-Quick staining of cytospin slides), there was a difference in the bactericidal capacity of each population. Phagocytic capacity in peritoneal exudate cells by live time-lapse imaging shown in figure 4A was greater than 75-fold higher in cells from mild cecal ligation and puncture control than in those from severe cecal ligation and puncture control (fig. 4B). Therapeutic interferon- $\beta$  administration restored the bactericidal function significantly, whereas the prophylactic administration of interferon- $\beta$  did not (fig. 4B).

In the severe cecal ligation and puncture therapeutic interferon- $\beta$  group compared with severe cecal ligation and puncture control, intraperitoneal/systemic cytokines were increased, and in particular, serum KC, serum/peritoneal MCP1, and serum  $\text{TNF}\alpha$  showed statistical significance (fig. 5, A and B). In contrast, the severe cecal ligation and puncture prophylactic interferon- $\beta$  group had comparable to less cytokines/chemokines compared with severe cecal ligation and puncture control (fig. 5B).

### Effects of Severe Sepsis on Circulating Neutrophils and Therapeutic Efficacy of Interferon- $\beta$

Because the systemic administration of therapeutic interferon- $\beta$  was associated with improved survival and increased numbers of recruited neutrophils in the peritoneal cavity, we evaluated the cell surface expression of chemokine receptors on circulating blood neutrophils. Complete blood count including leukocyte count, platelet count, and hemoglobin concentration were not dramatically different among





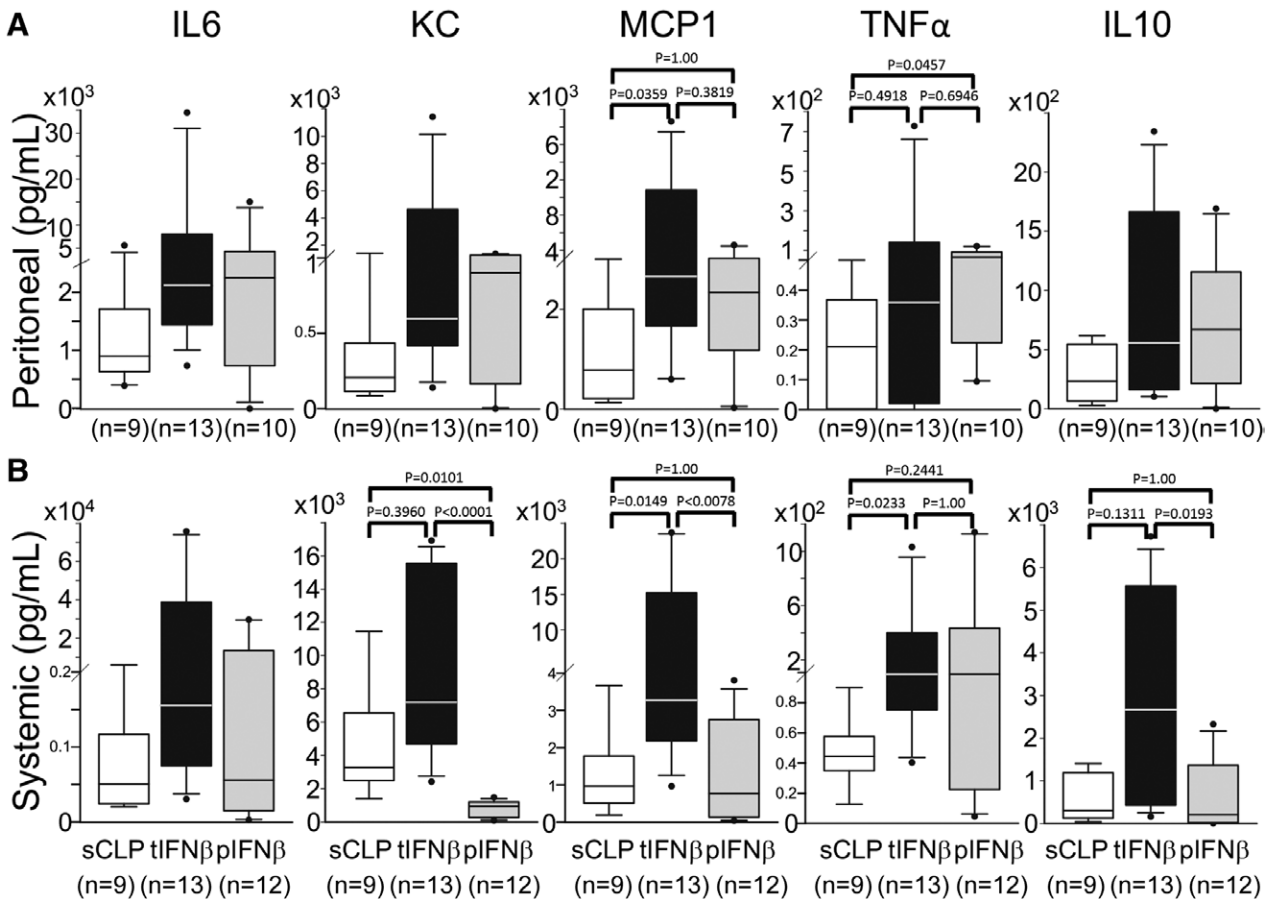
**Fig. 4.** Severe sepsis is associated with the significantly impaired bactericidal capacity of peritoneal exudate cells and is partially restored by therapeutic (t) interferon (IFN)- $\beta$ . Overall,  $2.5 \times 10^4$  cells/100  $\mu$ l of peritoneal exudate cells were incubated with 50  $\mu$ g/ml of pH-sensitive fluorescent dye-conjugated *Escherichia coli* bioparticles, and time-sequence phagocytic capacity was evaluated with IncuCyte ZOOM (Essen Bioscience, Inc., USA). (A) Time-course changes in fluorescent intensity for representative mice from each group. Open triangle, mild cecal ligation and puncture (mCLP) control (ctrl); open circle, severe cecal ligation and puncture (sCLP) ctrl; closed circle, sCLP with tIFN $\beta$ ; and gray circle, sCLP with prophylactic (p) IFN $\beta$ . (B) Summary of values in each group at 18 h. Hatched bar, mild cecal ligation and puncture control; open bar, severe cecal ligation and puncture control; closed bar, severe cecal ligation and puncture with therapeutic IFN $\beta$ ; gray bar, severe cecal ligation and puncture with prophylactic IFN $\beta$ . Data are expressed as a box plot; middle line indicates median, upper/lower box lines indicate 75/25 percentiles, and upper/lower error bars indicate 90/10 percentiles, respectively. Top and bottom dots indicate maximum and minimum values, respectively. Numbers (n) of mice included for each group are indicated in parentheses.

groups, although a statistically significant decline in platelet count was observed in severe cecal ligation and puncture control and severe cecal ligation and puncture prophylactic interferon- $\beta$  compared to mild cecal ligation and puncture control (Supplemental Digital Content 4, <http://links.lww.com/ALN/B679>). CXC chemokine receptor-2 expression on the surface of peripheral blood neutrophils was significantly suppressed in mild cecal ligation and puncture control compared with healthy control mice ( $P = 0.0181$ ) and mild cecal ligation and puncture control mice ( $P = 0.0008$ ), indicating that responses of neutrophils to chemokines during severe peritonitis may be reduced. CXC chemokine receptor-2 expression was restored by the systemic administration of interferon- $\beta$  12 h after cecal ligation and puncture induction (fig. 6). Systemic interferon- $\beta$  before cecal ligation and puncture showed significantly less CXC chemokine receptor-2 expression compared with mild cecal ligation and puncture control.

#### Effect of Lipopolysaccharide Exposure on Peritoneal Macrophage Function and Therapeutic Efficacy of Interferon- $\beta$

To further explore the mechanism of impaired innate immunity observed *in vivo*, bactericidal capacity of macrophages with or without lipopolysaccharide and/or interferon- $\beta$  was evaluated *in vitro*. The phagocytic capacity of peritoneal macrophages isolated from healthy control mice was increased when stimulated with lipopolysaccharide for 3 h, but was reduced significantly at 12 and 24 h later. When

interferon- $\beta$  was added in the medium 12 h later and cells incubated for another 12 h, the phagocytic capacity returned to the same levels as cells stimulated with lipopolysaccharide for 3 h (fig. 7). A murine peritoneal macrophage-derived cell line, RAW264.7, showed increased IL-6 messenger RNA (mRNA) expression upon stimulation for 3 h with 100 ng/ml lipopolysaccharide, peaked at 12 h, and declined 24 h later (Supplemental Digital Content 5, <http://links.lww.com/ALN/B680>). Suppressor of cytokine signaling 3 mRNA concentrations were increased and maintained after 24-h incubation (Supplemental Digital Content 5, <http://links.lww.com/ALN/B680>). After 12-h incubation with lipopolysaccharide, adding interferon- $\beta$  increased lipopolysaccharide-induced IL-6 mRNA expression and protein secretion (fig. 8, A and B) despite sustained increases in suppressor of cytokine signaling 3 and p21 (fig. 8, C and D), indicating cells were in a lipopolysaccharide-tolerant phase.<sup>30,31</sup> In contrast, previous incubation with interferon- $\beta$  failed to increase IL-6 mRNA and protein by subsequent stimulation with lipopolysaccharide (fig. 8, A and B). Expressions of suppressor of cytokine signaling 3 and p21 were further enhanced by previous incubation with interferon- $\beta$  followed by lipopolysaccharide stimulation (fig. 8, C and D). These results indicate that sustained stimulation with lipopolysaccharide induced a time-dependent decline in phagocytic capacity, cytokine secretion, and mRNA expression of inflammatory cytokines and that interferon- $\beta$  restored them when administered later. Previous administration of interferon- $\beta$  failed



**Fig. 5.** Cytokine expression is increased in the severe (s) cecal ligation and puncture (CLP) with therapeutic (t) interferon (IFN)- $\beta$  group compared with the sCLP controls. Cytokine concentrations in peritoneal lavage fluid (A) and serum (B) collected at 24 h after CLP in mice with sCLP control (white boxes), sCLP with tIFN $\beta$  (closed boxes), or sCLP with prophylactic (p) IFN $\beta$  (gray boxes). Data are expressed as a box plot; middle line indicates median, upper/lower box lines indicate 75/25 percentiles, and upper/lower error bars indicate 90/10 percentiles, respectively. Top and bottom dots indicate maximum and minimum values, respectively. Numbers (n) of mice included for each group are indicated in parentheses.

to restore impaired functions and increased Toll-like receptor 4 signal inhibitory pathways.

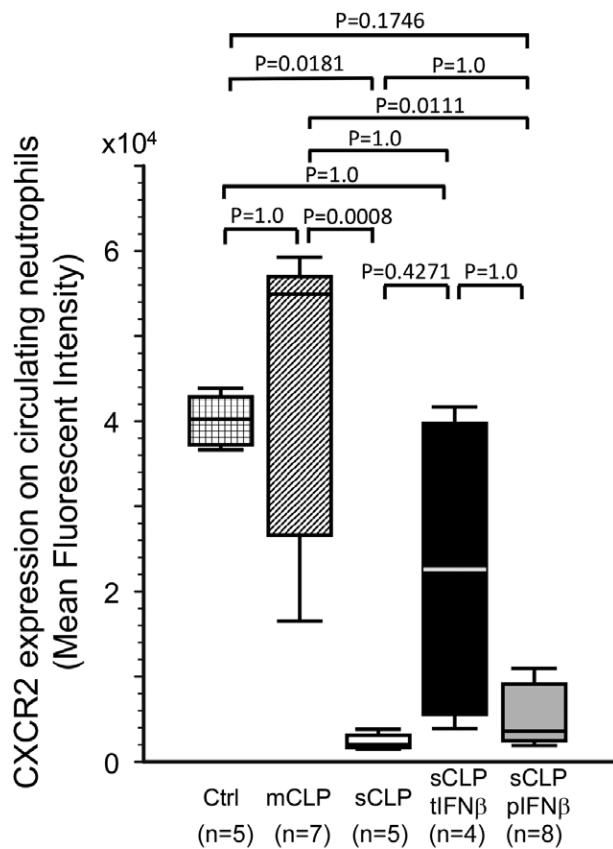
## Discussion

Severe peritoneal sepsis induced by cecal ligation and puncture with an 18-gauge needle was associated with impaired innate immunity 24 h after cecal ligation and puncture and a lower survival rate compared with mild peritonitis induced with a 22-gauge needle. Systemic interferon- $\beta$  12 h after severe peritonitis onset restored impaired innate immune function, increased peritoneal or systemic cytokine concentrations, and improved survival. However, interferon- $\beta$  administration before the onset had no therapeutic benefit. Sustained stimulation of macrophages with lipopolysaccharide induced suppressor of cytokine signaling 3 and p21 and induced cells into a lipopolysaccharide-tolerant phase. Interferon- $\beta$  restored impaired IL-6 expression even in lipopolysaccharide-tolerant cells. Previous exposure to interferon- $\beta$  increased suppressor of cytokine signaling 3 and p21 expressions but not lipopolysaccharide-induced IL-6 expression.

## Innate Immune Suppression Occurs in the Early Phase of Severe Sepsis

Past clinical trials to block the initial hyperinflammatory, cytokine-mediated phase of sepsis (neutralizing antibodies against endotoxin or TNF $\alpha$ , soluble receptor antagonist against IL-1) had no clinical efficacy to improve survival.<sup>13,15,31</sup> Improved treatment protocols succeeded in rescuing most sepsis patients from the initial hyperinflammatory phase, and thus the current main cause of death from sepsis is a failure to control the primary infection or the acquisition of secondary opportunistic infections.<sup>32</sup> Occurrence of secondary infection is associated with increased mortality,<sup>33</sup> and hypoimmune states were more likely in nonsurvivors of sepsis,<sup>18</sup> indicating defects in host-defense mechanisms are closely associated with mortality from sepsis. Under these circumstances, immunopotentiating therapies have begun to gain attention.

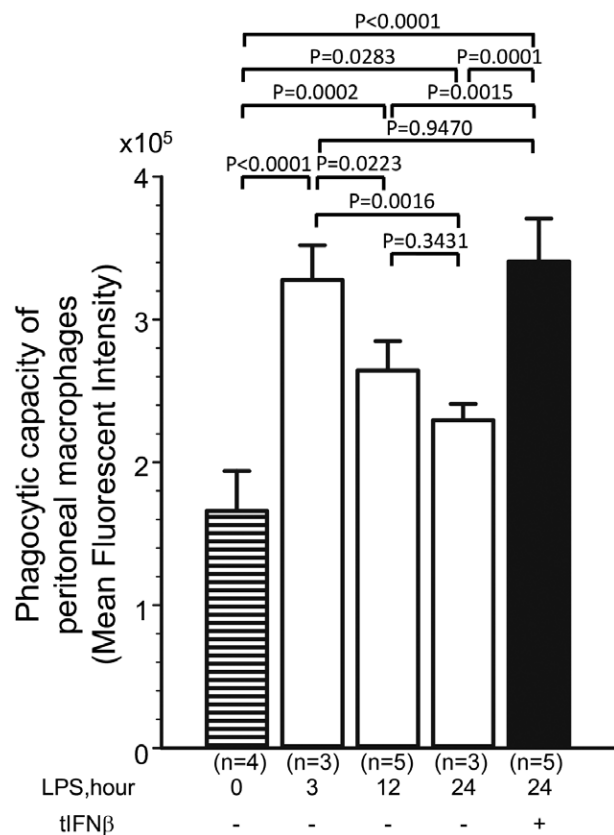
Formerly, the sepsis concept suggested the initial hyperinflammatory state was followed by an immunosuppressive state.<sup>15,16,32</sup> However, a hypoimmune state was reported to occur in the early phase of severe sepsis with poor outcomes.<sup>33</sup>



**Fig. 6.** CXC chemokine receptor (CXCR)-2 expression on circulating neutrophils is reduced after severe (s) cecal ligation and puncture (CLP) and is restored by therapeutic (t) interferon (INF)- $\beta$  but not by prophylactic (p) IFN $\beta$ . CXCR2 expression on Ly6G $^{+}$  neutrophils in heparinized whole blood collected 24h after CLP was evaluated by flow cytometry. Box with check pattern, normal control (ctrl); hatched box, mild (m) CLP control; open box, severe CLP control; closed box, severe CLP with therapeutic IFN $\beta$ ; gray box, severe CLP with prophylactic (p) IFN $\beta$ . Data are expressed as a box plot; middle line indicates median, upper/lower box lines indicate 75/25 percentiles, and upper/lower error bars indicate 90/10 percentiles, respectively. Numbers (n) of mice included for each group are indicated in parentheses.

In an animal study, immune suppression occurred 4 days after sepsis onset induced by sublethal cecal ligation and puncture<sup>29</sup>; however, Chiswick *et al.* reported a much earlier occurrence in severe cecal ligation and puncture models.<sup>34</sup> Therefore, the severity of sepsis might affect the timing of immunosuppression.

In the current study, the antimicrobial ability of peritoneal phagocytes 24h after cecal ligation and puncture was demonstrated in mild peritonitis, but was highly suppressed in severe peritonitis with high mortality. The current study suggests intraperitoneal immune suppression, where immune suppressive therapy is ineffective or causes harm, occurs within 24h in mice with severe septic peritonitis, indicating the risk of initiating immunomodulatory (*i.e.*, immune suppressive) therapy even at the early phase of sepsis.

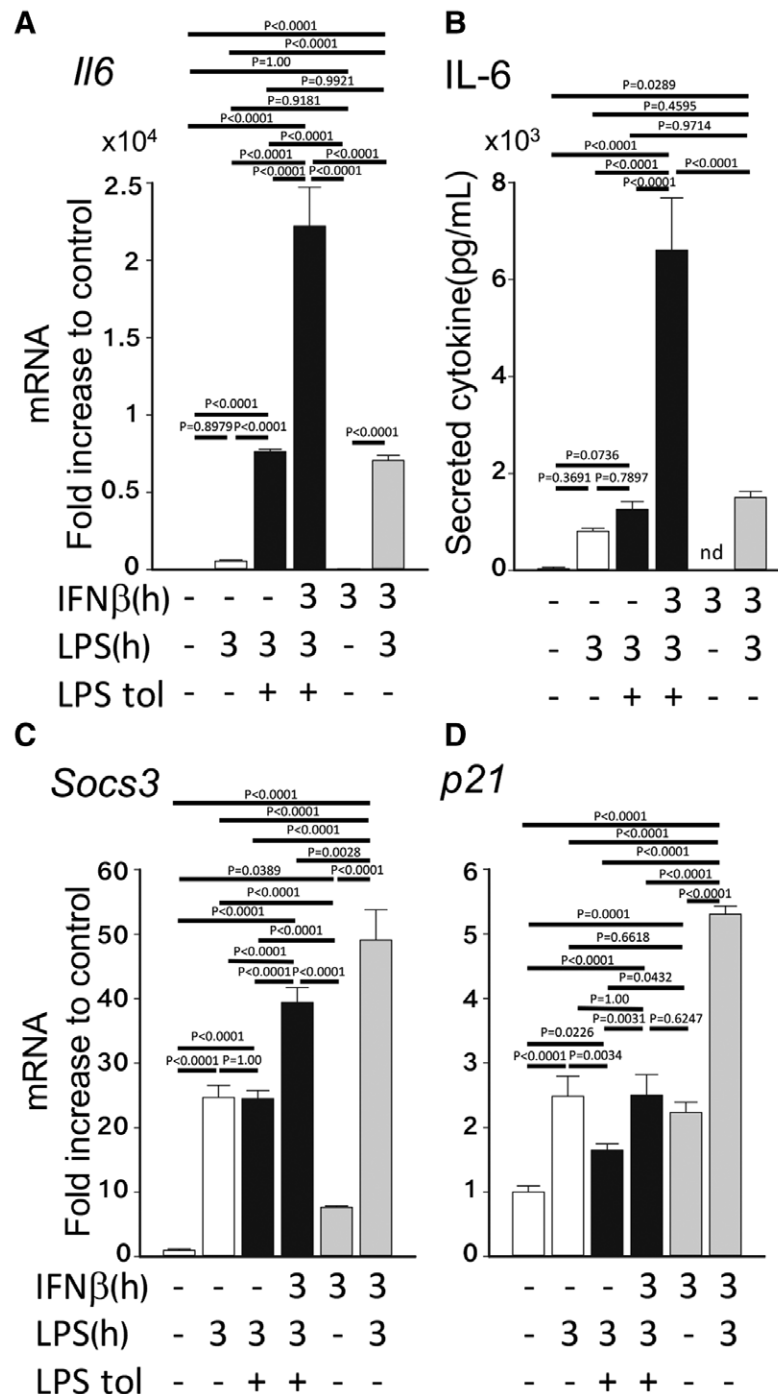


**Fig. 7.** Time course decline of phagocytic capacity of peritoneal macrophages stimulated with lipopolysaccharide (LPS) and restored by the later addition of interferon (IFN)- $\beta$  (1,500 U/ml). Peritoneal macrophages from normal mice were seeded at  $8 \times 10^5$  cells/500  $\mu$ l of Roswell Park Memorial Institute media with L-glutamate, 25mM HEPES supplemented with 10% fetal bovine serum/well and incubated with LPS (10ng/ml) for the indicated times. After the designated period of incubation, phagocytic capacity was evaluated by adding fluorescent latex beads for another 24h. Fluorescent intensity of harvested cells was measured by flow cytometry. In some wells, IFN $\beta$  was added 12h after LPS stimulation and incubated for a further 12h. Horizontal bar, without LPS; open bar, stimulated with LPS for the indicated period; and closed bar, stimulated with LPS for 24h and coincubation with IFN $\beta$ . Data are shown as the mean ( $\pm$ SD), and numbers (n) of mice included for each condition are indicated in parentheses.

### Possible Molecular Mechanism of Immune Suppression in Severe Bacterial Infection and Biochemical Role of Interferon- $\beta$

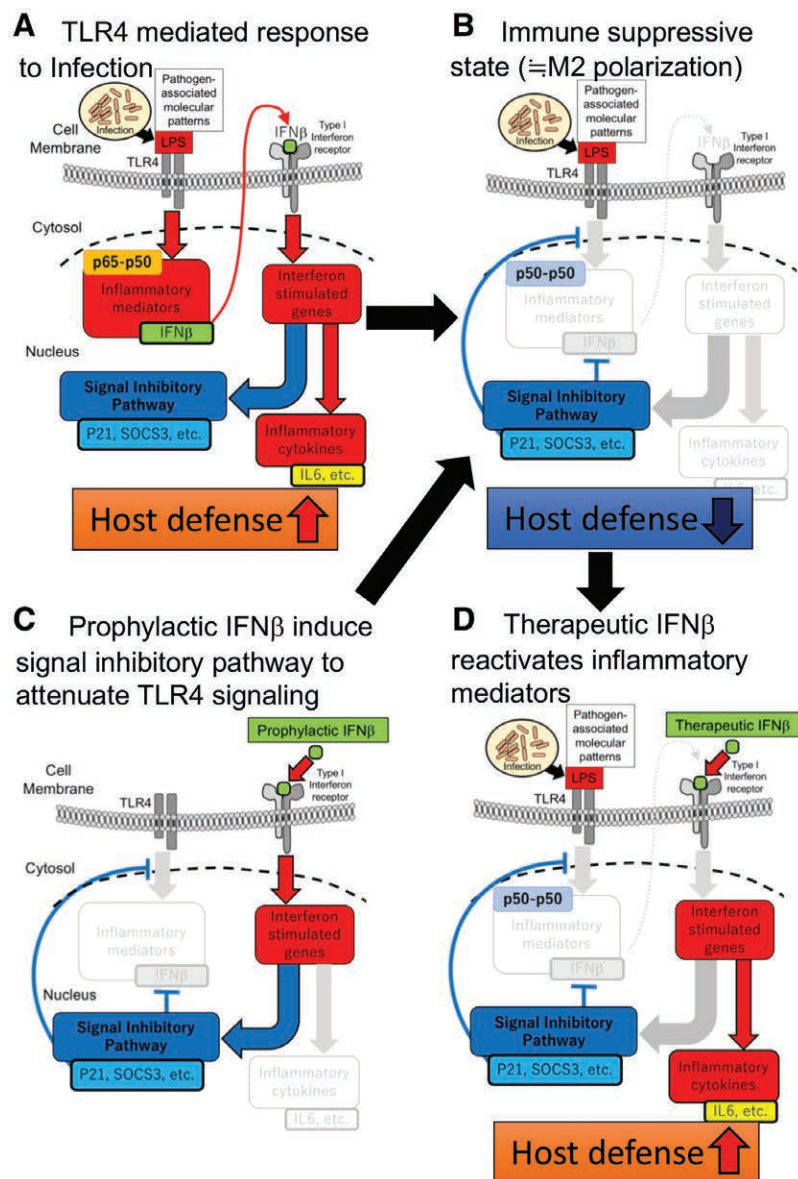
A reduction in phagocytic capacity may be caused by the exhaustion of cells in a severely infected site or by mediators that suppress cell function. In our model, the viability of collected peritoneal exudate cells was greater than 99%, and the functions of these cells were improved with systemic interferon- $\beta$ . Therefore, the functional decline of phagocytes may be caused by immunosuppressive mediators rather than cell exhaustion, as previously reported.<sup>34</sup>

One mechanism of impaired innate immune function during severe infection is lipopolysaccharide tolerance, a well-described phenomenon describing weakened or absent



**Fig. 8.** Timing of interferon (IFN)- $\beta$  administration affects cellular responses to lipopolysaccharide (LPS). The murine peritoneal macrophage cell line, RAW264.7, in the culture media was stimulated with 100 ng/ml of LPS. IL6 mRNA expression (A) and IL-6 protein secretion (B), Socs3 mRNA (C) and p21 mRNA (D) were evaluated. Quantification of mRNA has done by real-time polymerase chain reaction using TaqMan gene expression assays (Applied Biosystems, USA) with StepOne Real-Time PCR System, and IL-6 level in the culture media was measured by enzyme-linked immune sorbent assay. Expression values for each gene were calculated by normalization to *Gapdh*, and fold change in expression to unstimulated control was determined for each condition. Numbers for each bar indicate the duration of stimulation with IFN $\beta$  or LPS. LPS tol (+) indicates cells incubated for 12h before stimulation with either or both IFN $\beta$  and LPS. nd indicates not done. When LPS-tolerant cells were stimulated with LPS, coincubation with IFN $\beta$  showed a marked increase in IL6 mRNA expression and secretion (A and B, right closed bar in the middle) compared with those without IFN $\beta$  (A and B, left closed bar in the middle), whereas previous administration with IFN $\beta$  before LPS stimulation did not (A and B, right gray bar). Patterns of expression of Socs3 and p21 are similar to IL6 in LPS-tolerant cells (C and D, closed bars in the middle). However, incubation with IFN $\beta$  increased the expression of Socs3 and p21 (C and D, second right gray bar), and additional LPS markedly increased their expressions (C and D, first right gray bar). Data are shown as the mean of three determinants ( $\pm$ SD).





**Fig. 9.** Hypothetical mechanism of sepsis-related immune suppression in macrophages and possible mechanism of therapeutic efficacy of interferon (IFN)  $\beta$ —based on previous publications and the current study. (A) Lipopolysaccharide (LPS) signaling of naive cells. Toll-like receptor (TLR) 4 signaling initially activates TLR4-related intracellular signaling including the p65-p50 nuclear factor- $\kappa$ B-mediated activation of downstream signaling for proinflammatory mediators including IFN $\beta$ . IFN $\beta$  binds to type 1 IFN receptor in an autocrine manner to activate the Janus kinase-signal transducer and activator of transcription pathway for the downstream expression of IFN-stimulated genes that encode IL-6. (B) LPS-tolerant cells. Concurrently, suppressors of cytokine signaling, such as suppressor of cytokine signaling 3 (Socs3) and p21, were expressed and attenuated subsequent TLR4 signaling. A p21-induced shift of the nuclear factor- $\kappa$ B subunits from p65-p50 to p50-p50 inhibits the nuclear translocation of nuclear factor- $\kappa$ B to bind to nuclear factor- $\kappa$ B-inducible genes, including IFN $\beta$ . These phenomena are termed M2 polarization. (C) Prophylactic IFN $\beta$  might prime macrophages and concomitantly induce Socs and p21 genes to induce macrophages irresponsive to TLR4 signaling (i.e., M2 polarization), which will be compromised to subsequent overwhelming infection (B). (D) Therapeutic IFN $\beta$  might reactivate downstream IFN $\beta$  signaling to increase the expression of inflammatory cytokines/chemokines and elicit the bactericidal capacity of the host. See text for further details.

macrophage and monocyte reactivity against lipopolysaccharide when preexposed to a sublethal dose of lipopolysaccharide.<sup>35</sup> This is homologous to the macrophage polarization from M1 to M2 phenotype,<sup>36–38</sup> which occurs during the immunosuppressive state observed in sepsis and severe infection.

Lipopolysaccharide tolerance in macrophages is mediated by multiple factors including suppressor of cytokine signaling 1 and 3<sup>35</sup> and a shift of nuclear factor- $\kappa$ B subunit p65-p50 predominance to p50-p50 mediated by p21 expression<sup>39</sup> (fig. 9, A and B, integrated and simplified to blue line).

Interferon- $\beta$  plays an essential role in Toll-like receptor 4 signaling to elicit M1 responses to infection. Type 1 interferon transfers the lipopolysaccharide signal downstream through interferon- $\beta$  autocrine induction to induce various inflammatory mediators that exert antibacterial actions (fig. 9A, indicated in red).<sup>40</sup> Concurrently, type 1 interferon signals induce suppressor of cytokine signaling 1 and 3 and p21, which form a negative feedback loop to restrict the magnitude and duration of Toll-like receptor 4 signaling (fig. 9, A and B, indicated in blue).<sup>38,41,42</sup> Furthermore, p21-induced p50-p50 predominance inhibits autocrine interferon- $\beta$  expression. The current results showed sustained increases in suppressor of cytokine signaling 3 and p21 after stimulation with lipopolysaccharide in contrast to a time-course decline of IL-6 mRNA in macrophages (Supplemental Digital Content 5, <http://links.lww.com/ALN/B680>), indicating a lipopolysaccharide-tolerant phenotype. Taken together, suppression of interferon- $\beta$  expression might be involved in M2 polarization and subsequent impairment of innate immunity during severe infection.

#### **Possible Interferon- $\beta$ Therapeutic Mechanism of Action**

For lipopolysaccharide-tolerant macrophages, exogenous interferon- $\beta$  increased IL-6 mRNA concentrations and IL-6 protein secretion despite sustained increases in suppressor of cytokine signaling 3 and p21 (fig. 8). Therapeutic administration of interferon- $\beta$  may reactivate type 1 interferon signaling and restore macrophage effector functions (*i.e.*, phagocytosis; fig. 4) and regulatory functions (cytokine/chemokine secretion; fig. 5), which contribute to improved survival (fig. 9D).

#### **Importance of Host Immune Status for the Effect of Interferon- $\beta$ Administration**

In contrast to improved host defense and survival in mice receiving therapeutic interferon- $\beta$ , prophylactic interferon- $\beta$  administration did not improve host defense, but rather increased mortality. Phagocyte functions and peritoneal and systemic cytokine responses in the prophylactic interferon- $\beta$  group were comparable to the severe cecal ligation and puncture controls. Prophylactic interferon- $\beta$  administration had a detrimental effect on survival, inconsistent with a report by Yoo *et al.* that showed pretreatment with 1,000 U/20g interferon- $\beta$  improved survival after cecal ligation and puncture.<sup>30</sup> In their model, they applied 24-gauge needles with 75% cecum ligation that should induce a milder and sustained infection model compared with our cecal ligation and puncture control. Although they did not evaluate innate immune function, it is anticipated that hyperimmune response may take place just like in our mild cecal ligation and puncture control group. In that case, pretreatment with interferon- $\beta$  may work to optimize immune status and avoid the exaggerated immune response that may damage intact organs and increase the mortality rate. In contrast, our murine model is an acute severe peritoneal infection with

larger holes that require activation of a host defense mechanism. In the current study, preincubation with interferon- $\beta$  increased suppressor of cytokine signaling 3 and p21 mRNA expressions, and the later addition of lipopolysaccharide increased this further (fig. 8, C and B) and impaired IL-6 expression (fig. 8, A and B). Therefore, induction of Toll-like receptor 4–signaling inhibitors such as suppressor of cytokine signaling 3 and p21 induced by previous exposure to exogenous interferon- $\beta$ <sup>43,44</sup> may inhibit Toll-like receptor 4 downstream signaling by subsequent cecal ligation and puncture, and thus peritoneal macrophages cannot respond to the infection as an M1 phenotype (fig. 9, C and B, indicated in blue). Based on the current data and past publications, the effects of interferon- $\beta$  may be bidirectional, depending on the innate immune status of the host and the magnitude of insult that affects innate immune status.

#### **Clinical Implications**

This study suggests systemic interferon- $\beta$  may be therapeutic for severe peritoneal sepsis even when it is administered after the onset of peritonitis. A previous open-label clinical trial of interferon- $\beta$  on acute respiratory distress syndrome by Bellington *et al.* showed improved survival.<sup>23</sup> They speculated the mechanism of action was improved vascular barrier function. In addition to that, because 70% of their patients with acute respiratory distress syndrome were complicated with infection,<sup>23</sup> interferon- $\beta$  may improve their host defense, which might improve their prognosis. We recently demonstrated functional impairment of alveolar macrophages in mild peritoneal sepsis of mice, which is restored by systemic interferon- $\beta$ .<sup>24</sup> In actual clinical practice, infection is completed at the diagnosis of sepsis, and interferon- $\beta$  may be a promising candidate to treat patients suffering from ongoing severe infection.

However, because interferon- $\beta$  has detrimental effects depending on the timing of administration, it is important to carefully consider the optimal timing for its application. This might be achieved by assessing macrophage and neutrophil functions, especially the CXCR2 chemokine receptor-2 expression of circulating neutrophils as a surrogate marker for their immune status to predict the therapeutic effect of interferon- $\beta$ . Intracellular signal-inhibiting mediators such as p21 and suppressor of cytokine signaling 3 may be other candidates to indicate the optimal therapeutic window.

#### **Limitations**

Study limitations included not establishing a dose-response relationship or evaluating the timing, frequency, or route of interferon- $\beta$  administration to optimize therapeutic benefit. Nor did it define the temporal envelope of innate immune suppression and vulnerability to infection *in vivo*. Although we focused on innate immune function in response to severe infection, other vital organ functions should also be evaluated to explain the cause of increased survival for mice receiving interferon- $\beta$ . Elucidating the effect of interferon- $\beta$  on other immune cell functions (natural killer cells, lymphocytes) is mandatory.

## Conclusions

The temporary decline of the bactericidal capacity of innate immune cells was observed within 24 h during severe abdominal infection. Systemic interferon- $\beta$  was associated with the restoration of the host defense capacity of innate immune cells and improved the survival rate. The therapeutic administration of interferon- $\beta$  was effective even after severe infection was initiated; however, the prophylactic use of interferon- $\beta$  before the induction of peritonitis was ineffective.

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## Competing Interests

Drs. Uchida and Yamada had a collaborative research agreement with Maruishi Pharmaceutical Co. Ltd., Osaka, Japan (2012–2015). Maruishi Pharmaceutical Co. Ltd. provided the interferon- $\beta$  used in this study. The remaining authors declare no competing interests.

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